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# Ultrasound-responsive thrombus treatment with zinc-stabilized gelatin nano-complexes of tissue-type plasminogen activator

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**Short title:** Ultrasound-responsive t-PA nano complexes

**Key words:** t-PA, thrombolytic therapy, gelatin, metal ions, ultrasound

## ABSTRACT

This study is undertaken to design zinc-stabilized gelatin nano-complexes of tissue-type plasminogen activator (t-PA) for thrombolytic therapy where the t-PA activity can be recovered in the blood circulation upon ultrasound irradiation. Various molecular weights of gelatin were complexed with t-PA by their simply mixing in aqueous solution. Then, zinc acetate, calcium acetate or magnesium acetate was added to form nano-sized gelatin–t-PA complexes. The complexes had the apparent molecular size of about 100 nm. When zinc ions were added to the gelatin–t-PA complexes, the t-PA activity was suppressed most strongly to 57% of the original, free t-PA activity. Upon ultrasound exposure *in vitro*, the t-PA activity was fully recovered. A cell culture experiment with L929 fibroblasts demonstrated no cytotoxicity of complexes at the concentration used for the *in vivo* experiment. The half-life of t-PA in the blood circulation prolonged by the complexation with gelatin and zinc ions. The zinc-stabilized t-PA–gelatin complex is a promising t-PA delivery system which can manipulate the thrombolytic activity by the local ultrasound irradiation.

## 1 INTRODUCTION

2 In acute myocardial infarction, treatment strategies aimed at rapidly restoring complete and  
3 persistent coronary flow, and the recanalisation of infarct artery is the target of both  
4 pharmacological and mechanical reperfusion techniques. Tissue-type plasminogen activator  
5 (t-PA) is widely used as a thrombolytic drug because of its high fibrin specificity (Armstrong &  
6 Collen, 2001) and efficacy if administered early. However, t-PA has some adverse effects to be  
7 clinically resolved, including the risk of allergic reactions, bleeding complications, and short  
8 half-life (Blann et al., 2002; Bode et al., 1996; Turpie et al., 2002), which often limits the  
9 therapeutic applications. In fact, among patients treated with plasminogen activators,  
10 approximately 20% showed the adverse effect of bleeding (Chung et al., 2008). Therefore, the  
11 targeted delivery of thrombolytic drugs only to the necessary site is an attractive strategy to  
12 avoid such side effects. Drug delivery system (DDS) is a technology or methodology to  
13 efficiently delivery drugs to the site of action by making use of carrier materials. For the  
14 thrombolytic applications, several investigations on the delivery of t-PA have been reported  
15 (Chung et al., 2008; Tiukinhoy-Laing et al., 2007; Shaw et al., 2009; Heeremans et al., 1995;  
16 Smith et al., 2010; Molina et al., 2006; Collis et al., 2010).

17 Recently, an increasing interest has been expressed in intelligent DDS that has the capacity  
18 for triggered release by external factors, such as ultrasound, temperature, pH, or  
19 electromagnetic fields (Tiukinhoy-Laing et al., 2007; Rapoport et al., 2002; Marin et al., 2001;  
20 Daocheng and Mingxi, 2008; Frinking et al., 1998; Huang and MacDonald, 2004). Among  
21 these factors, ultrasound is one of the attractive methodologies to control drug release, as it is  
22 non-invasive and can deeply penetrate into the interior of body. Several systems of  
23 ultrasound-triggered drug release, specifically the poly (DL lactide-co-glycolide) system  
24 (Frinking et al., 1998) and acoustically active liposomes (Huang and MacDonald, 2004), have  
25 been reported. However, all of the ultrasound triggered release systems cannot be used



1 clinically because of their low sensitivity in response to ultrasound stimulation and the poor  
2 drug loading.

3 To date, we have explored various DDS carriers of gelatin. Gelatin has been extensively  
4 used for industrial, pharmaceutical, and medical applications, and the bio-safety has been  
5 proved through the long clinical usages as surgical biomaterials and drug ingredients (Veis,  
6 1965). Biodegradable hydrogels of gelatin and the derivatives have been designed for the  
7 controlled release of various drugs, such as basic fibroblast factor (bFGF) (Ikada and Tabata,  
8 1998), bone morphogenetic protein-2 (BMP-2) (Yamamoto et al., 1998), transforming growth  
9 factor beta 1 (TGF- $\beta$ 1) (Yamamoto et al., 2000), hepatocyte growth factor (HGF) (Ozeki et al.,  
10 2001), NK4 plasmid DNA (Kushibiki et al., 2004a), matrix metalloproteinase-1 (MMP-1)  
11 plasmid DNA (Aoyama et al., 2003; Lin et al., 2009), small interfering RNA (Nakamura et al.,  
12 2008), simvastatin (Tanigo et al., 2010), and cisplatin (Konishi et al., 2003). In addition, gelatin  
13 and the water-soluble chemical derivatives have been used to improve the *in vivo* stabilization  
14 and targeting of drugs (Kushibiki and Tabata, 2005) as well as form polymer micelles for drug  
15 delivery (Tanigo et al., 2010). On the other hand, metal ions, such as  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Sr}^{2+}$   
16 ions, have been used as the cross-linker for nanoparticles preparation (Jay and Saltzman, 2009;  
17 Jay et al., 2008; Mørch et al., 2006). In addition, about 2 g of zinc ions are known to be present  
18 in the adult human body and the ions play an important role in the activity of many metal and  
19 metal-activated enzymes (Krebs, 2000).

20 In this study, a novel delivery system for t-PA where the activity is locally switched on by  
21 ultrasound was designed, and the thrombolytic effects were evaluated. As the t-PA carrier, the  
22 gelatin and metal ions were used. The complexes of t-PA with the gelatin and metal ions were  
23 characterized in terms of the physicochemical properties and the thrombolytic activity. The  
24 t-PA activity of complexes was examined before and after ultrasound exposure to assess the  
25 potential of ultrasound-induced activity recovery. We examine the retention in the blood

circulation and tissue distribution of t-PA complexes administered intravenously.

## MATERIALS AND METHODS

### *Materials*

Gelatin samples with an isoelectric point (IEP) of 9.0 (weight-averaged molecular weight ( $M_w$ ) = 2,000, 3,000, 5,000, 20,000, 50,000, and 100,000) prepared by an acid process of pig skin type I collagen, were kindly supplied from Nitta Gelatin Inc., Osaka, Japan. Zinc acetate ( $\text{Zn}(\text{CH}_3\text{COO})_2$ ) solution, magnesium acetate ( $\text{Mg}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$ ), and calcium acetate ( $\text{Ca}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$ ) were purchased from Nacalai Tesque Inc., Kyoto, Japan. t-PA (Cleactor<sup>®</sup> administration 1,600,000) was purchased from Eisai Co., Ltd., Tokyo, Japan. Chromozym t-PA was purchased from Roche Diagnostics, Germany. Plasmin, plasminogen, and thrombin isolated from the human plasma were obtained from EMD Biosciences Inc., CA. Plasminogen-free fibrinogen from the human plasma was purchased from Sigma-Aldrich Corp., MO. Other chemicals of the purest quality were obtained.

### *t-PA assay with a peptidic substrate*

The hydrolytic activity of t-PA was determined by the conventional assay with a synthetic substrate (Chromozym t-PA, Roche Diagnostics, Germany). Briefly, 20  $\mu\text{l}$  of t-PA solution and 20  $\mu\text{l}$  of different concentrations of zinc acetate were mixed and incubated at 37°C for 30 min. Then, 15  $\mu\text{l}$  of the mixture was added to 150  $\mu\text{l}$  of Chromozym t-PA solution containing 90 mM Tris buffer (pH 8.5) and 0.14 wt% Tween 80. After incubation at 37°C for 30 min, the reaction was stopped by addition of 75  $\mu\text{l}$  of 10 vol% citric acid. The absorbance at 405 nm of the reaction mixture (200  $\mu\text{l}$ ) was measured on a microplate reader (VERSAmix, Molecular Devices Co., Ltd., Japan).

1     *Preparation of t-PA complexes with gelatin and metal ions*

2         To prepare the complexes of t-PA and gelatin, 10 mg of gelatin with different molecular  
3     weights in 10 mM phosphate-buffered solution (PBS, pH 7.4) (0.25 ml) was mixed with 0.5 ml  
4     of PBS containing 0.5 mg of t-PA. After agitation at 37°C for 30 min, different concentrations  
5     of zinc acetate, magnesium acetate, or calcium acetate (0.25 ml) were added to the mixture,  
6     followed by agitation at 37°C for 30 min to prepare the complex of t-PA with gelatin and metal  
7     ions.

9     *Measurement of apparent molecular size of t-PA complexes with gelatin and zinc ions*

10        The apparent molecular size of t-PA complexes with gelatin and zinc ions was measured by  
11     dynamic light scattering (DLS) apparatus (DLS-7000, Otsuka Electronic, Osaka, Japan)  
12     equipped with an Ar<sup>+</sup> laser at a detection angle of 90° at 37°C. The autocorrelation function of  
13     samples was analyzed based on the histogram method and  $R_s$  value was calculated  
14     automatically by the equipped computer software. The experiments were done 3 times  
15     independently for each sample.

17     *Assay of t-PA activity by fibrin plate method*

18        To determine the *in vitro* thrombolytic activity of t-PA, a fibrin clot lysis assay was  
19     performed according to the conventional plasminogen-rich fibrin plate method (Astrup and  
20     Müllertz, 1952). Briefly, 0.4 wt% human fibrinogen solution (10 ml) in 0.17 M borate buffer  
21     (pH 7.8) was poured into a 10-cm petri dish (Flat). Plasminogen (0.2 ml, 50 U/ml) was added to  
22     the fibrinogen solution. Clotting was induced by adding 0.2 ml of human thrombin (100 U/ml),  
23     followed by leaving for 30 min at room temperature to allow the clot formation. Then, 2 µl of  
24     PBS solution containing t-PA or t-PA complexes with gelatin and metal ions was carefully  
25     placed onto the plate. After incubation at 37°C for 1 hr, the diameter of clear zones was

measured while the standard curve between the diameter and the activity was prepared using human plasmin at determined concentrations. The percent activity was calculated as the percentage of sample activity to that of free t-PA. The experiment was done at least 3 times independently for each sample.

To evaluate the t-PA activity in bovine serum albumin (BSA) solution, the similar assay procedure was performed except that various solutions of t-PA complex were incubated with 42 mg/ml of BSA for 90 min at 37°C before the t-PA activity assay.

To evaluate the t-PA activity before and after ultrasound irradiation *in vitro*, various solutions of t-PA complex were treated with continuous wave ultrasound at 1 MHz with an intensity of 0.75 W/cm<sup>2</sup> for 5 min. Then, the t-PA activity of solution exposed to ultrasound was similarly evaluated to compare with that of untreated solution.

### Cell viability

Cytotoxicity was assayed for mouse fibroblast L929 cells by using a cell counting kit (Nacalai Tesque Inc., Kyoto, Japan). The cells were seeded on each well of 96-well cell culture dish (Corning Inc., NY) at a density of 5000 cells/100 µl per a well and cultivated in Eagle's MEM (E-MEM) medium containing 10 vol% fetal calf serum (FCS) for 24 hr at 37°C in 5% CO<sub>2</sub> –95% air atmosphere. The medium was changed to 90 µl of the fresh medium, and then 10 µl of PBS solution containing complexes of t-PA with gelatin and zinc ions was applied to each well, followed by incubation for 48 hr. Then, the medium was changed to 100 µl of E-MEM medium containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) and incubated further for 2 hr. The absorbance of medium was measured at 450 nm by the VERSAmax microplate reader (Molecular Devices, Sunnyvale CA). The percentage of cell viability was expressed as 100% for non-treated cells. The experiment was done 6 times

independently for each sample.

### *Body distribution tests of t-PA complexes with gelatin and zinc ions*

The body distribution of complexes of t-PA with gelatin and zinc ions was evaluated for 6-week-old female ddY mice (Simizu Laboratory Supplies Co., Ltd., Kyoto, Japan) as described previously (Kushibiki et al., 2004b). Briefly, t-PA was radioiodinated according to the chloramine T method (Wilbur et al., 1989). The t-PA complexes were similarly prepared by the procedure described above other than using  $^{125}\text{I}$ -labeled t-PA. The  $^{125}\text{I}$ -labeled t-PA and the complexes with gelatin and zinc ions were administered into the tail vein of mice in a PBS volume of 100  $\mu\text{l}$ . At different time intervals after administration, the blood samples were collected from the eye orbit. The radioactivity of blood was counted on the gamma counter (ARC-301B, Aloka, Tokyo, Japan). The percentage of radioactivity remaining was expressed as 100% for the radioactivity of t-PA initially administrated.

At 60 min after administration, the mice were sacrificed and their tissues and organs were excised and rinsed quickly with cold water to remove the surface blood. Then, the radioactivity of tissues and organs was counted as described above. The animal experiments were carried out according to the Institutional Guidance of Kyoto University on Animal Experimentation. The experiment was done 6 times independently for each sample at each time point.

### *Statistical analysis*

The statistical analysis was performed using an unpaired Student's *t* test. All the data were presented as the mean  $\pm$  the standard deviation (SD).

# 1 RESULTS

## 2 *Characterization of t-PA complexes with gelatin and zinc ions*

3 Figure 1 shows the apparent molecular size of gelatin with different molecular weights, the  
4 complexes of t-PA and zinc ions. The apparent molecular size of gelatins with molecular  
5 weights ranging from 2,000 to 5,000 could not be measured because of their too small size. The  
6 molecular weight size of gelatin tended to increase with the increased molecular weight. The  
7 apparent molecular size of gelatin was increased by the addition of zinc ions. Irrespective of the  
8 molecular weight of gelatin, the apparent molecular size was dramatically changed by mixing  
9 with t-PA and zinc ions. The size of t-PA complexes tended to decrease with an increase in the  
10 molecular weight of gelatin.

11

## 12 *Thrombolytic activity of t-PA complexes with gelatin and zinc ions*

13 Figure 2 shows the t-PA activity change by the complexation with gelatin of different  
14 molecular weights and zinc ions. Irrespective of the gelatin molecular weight, the thrombolytic  
15 activity of t-PA was significantly suppressed by the complexation with gelatin and zinc ions  
16 (Figure 2A). The suppression tended to become stronger with a decrease in the molecular  
17 weight of gelatin. Figure 2B shows the t-PA activity change of complexes with gelatins and  
18 zinc ions in the presence of BSA. Interestingly, the suppression was weaker with a decrease in  
19 the molecular weight of gelatin. From the viewpoint of activity suppression, the following  
20 experiment was performed for the gelatin with a molecular weight of 100,000. Figure 3 shows  
21 change in the t-PA activity by complexing with zinc ions and gelatin at different concentrations.  
22 The activity of t-PA complexes tended to decrease with an increase in the concentration of  
23 gelatin, and then leveled off at the gelatin concentrations of 10 mg/ml and higher. When  
24 fractionated by a gel filtration column, the t-PA containing ratio of t-PA complexes at the  
25 gelatin concentrations of 10 mg/ml was approximately 84%.

# *Effect of metal ions on thrombolytic activity of t-PA complexes with gelatin*

Figure 4 shows the activity change of t-PA by complexation with gelatin and various types of metal ions. The t-PA activity was significantly suppressed by complexing with gelatin and every metal ions tested in this study (Figures 4A-C), although the t-PA activity was not suppressed by complexation with gelatin alone (data not shown). The suppression extent increased in a dose-dependent manner. Among zinc, magnesium, and calcium ions, the thrombolytic activity of complexes was suppressed most strongly with zinc ions. In addition, the stability of t-PA complexes with magnesium and calcium ions in serum was lower than that with zinc ions (data not shown). After ultrasound exposure, the suppressed t-PA activity of gelatin and zinc ions complexes was recovered to the level of original t-PA activity at the zinc concentrations of 5 mM or lower (Figure 4D).

Figure 5 shows the activity of t-PA in the presence of zinc ions at different concentrations. No decrease in the t-PA activity was observed at the zinc concentrations of 5 mM and lower.

Figure 6 shows the t-PA activity change of complexes by addition of different concentrations of EDTA, metal chelator. The suppressed t-PA activity of complexes tended to increase with an increase in the concentration of EDTA.

## *Cell viability of t-PA complexes with gelatin and zinc ions*

Figure 7A shows the cytotoxicity of t-PA complexes with gelatin and zinc ions. The complexes showed over 90% cell viability at the concentrations of 200  $\mu$ g/ml and lower. However, the cell viability gradually decreased at the concentrations of 300  $\mu$ g/ml and higher. Zinc ions, gelatin, and t-PA alone showed over 90% cell viability at the concentrations of 50  $\mu$ M, 500  $\mu$ g/ml, 50  $\mu$ g/ml and lower, respectively (Figures 7B, C). The concentration of zinc ions, gelatin, and t-PA used for complexation in the *in vivo* experiment were 2  $\mu$ M, 4  $\mu$ g/ml, and 0.2  $\mu$ g/ml, respectively, which is much lower than those of cytotoxicity.

1    *Body distribution of t-PA and t-PA complexes with gelatin and zinc ions*

2        Figure 8A shows the percentage of radioactivity remaining in the blood circulation  
3    following intravenous administration of  $^{125}\text{I}$ -labeled t-PA and the complexes. The radioactivity  
4    remaining was significantly higher for the complexes of gelatin and zinc ions than that of free  
5    t-PA over the time range studied. The blood concentration of free t-PA was rapidly reduced  
6    after administration. Complexation with gelatin and zinc ions prolonged the blood half-life of  
7    t-PA about 3 times. Figure 8B shows the tissue distribution of t-PA complexes with gelatin and  
8    zinc ions or free t-PA. The similar body distribution pattern was observed between the two  
9    groups.

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# 1 DISCUSSION

2 Several DDS trials of t-PA have been reported for the thrombotic diseases of myocardial  
3 infarction and ischemic stroke with various carriers. Liposomes encapsulating t-PA  
4 (Tiukinhoy-Laing et al., 2007; Shaw et al., 2009; Heeremans et al., 1995; Smith et al., 2010), or  
5 poly-(lactide-co-glycolide) microspheres incorporating t-PA and coated with Arg-Gly-Asp  
6 peptide, which is a minimal cell-recognizable peptide of adhesion protein, significantly  
7 accelerated thrombolysis *in vitro* (Chung et al., 2008). Combination of t-PA with microbubble  
8 induced the acceleration of ultrasound-enhanced thrombolysis in a model of acute stroke  
9 (Molina et al., 2006; Collis et al., 2010).

10 This study is undertaken to design a novel ultrasound-responsive t-PA delivery system with  
11 gelatin and zinc ions. Gelatin has been used for the controlled release of various drugs, proteins,  
12 and genes because of the good biodegradability and biocompatibility (Veis, 1965; Ikada and  
13 Tabata, 1998; Yamamoto et al., 1998, 2000; Ozeki et al., 2001; Kushibiki et al., 2004a; Aoyama  
14 et al., 2003; Lin et al., 2009; Nakamura et al., 2008; Tanigo et al., 2010; Konishi et al., 2003).  
15 The thrombolytic activity of t-PA was suppressed by the complexation with gelatin and zinc  
16 ions under the optimal condition, and recovered only when exposed to ultrasound. This  
17 ultrasound-induced recovery of t-PA activity is an ideal system considering several problems to  
18 be resolved clinically, such as the bleeding complication and the short half-life *in vivo*.

19 t-PA is a globular protein with an molecular weight of about 70,000 (Rijken and Lijnen,  
20 2008; Hedstrom, 2002) and the zeta potential is negative (data not shown). Therefore, it is  
21 expected that the t-PA molecule can electrostatically interact with gelatin (IEP = 9.0). The  $K_D$   
22 value between gelatin and t-PA was measured to be  $7.01 \times 10^{-8}$  M by the quartz crystal  
23 microbalance analysis. For example about specific interaction, previous surface plasmon  
24 resonance analysis indicated that  $K_D$  between the collagen-binding domain (CBD) from  
25 *Clostridium histolyticum* collagenase and mini-collagen peptide was  $5.72 \times 10^{-5}$  M (Wilson et

al., 2003). These results suggested that the interaction of t-PA and gelatin molecules for complexation was stronger than that of specific interaction between the CBD and the collagen peptide. However, the t-PA activity was not suppressed by complexation with gelatin (data not shown). It is highly conceivable that the t-PA molecules do not always interact with gelatin in the manner to biologically suppress the t-PA activity. Therefore, metal ions were added to modify the interaction between the gelatin and t-PA molecules.

The analysis on the nonredundant proteins structures from the Protein Data Bank showed that the most abundant metal ion in the body is  $Mg^{2+}$ , followed by  $Zn^{2+}$ ,  $Ca^{2+}$ ,  $Fe^{2+}$ ,  $Mn^{2+}$ ,  $Cd^{2+}$ ,  $Cu^{2+}$ ,  $Co^{2+}$ , and  $Ni^{2+}$  (Dokmanić et al., 2008). Based on this, considering the toxicity nature (Yamamoto et al., 2004),  $Mg^{2+}$ ,  $Zn^{2+}$ , and  $Ca^{2+}$  ions were tested in this study (Figure 4). The t-PA activity of complexes was most strongly suppressed by the addition of  $Zn^{2+}$  ions. It is likely that the t-PA and gelatin molecules of complexes was modified by a coordinate bond and an ionic bond with  $Zn^{2+}$  ions, whereas they were done by an ionic bond with  $Mg^{2+}$  and  $Ca^{2+}$  ions. The apparent molecular size of gelatin was dramatically changed by t-PA and  $Zn^{2+}$  ions complexation. It tended to increase with a decrease in the molecular weight (Figure 1). The thrombolytic activity of t-PA tended to suppress more strongly by the complexion with gelatin and zinc ions when the molecular weight of gelatin decreased (Figure 2A). The SDS-PAGE analysis and atomic absorption photometer revealed that the complexes of gelatin and t-PA, when fractionated by a gel filtration column, were contained zinc ions (data not shown). Moreover, no decrease in the t-PA activity was observed at the zinc concentrations of 5 mM and lower (Figure 5). With gelatin molecules, the t-PA activity was suppressed at low concentrations of zinc ions, although the t-PA activity was maintained without gelatin. Additionally, the suppression extent of t-PA activity tended to increase with an increase in the concentration of gelatin (Figure 3). Taken together, we can say with certainty that the interaction between the t-PA and gelatin molecules can be reinforced by mixing zinc ions,

resulting in the suppressed biological activity. The amino acid residues contributing to the coordination bonds with zinc ions are Cys, His, Asp, and Glu in the metalloproteins (Dokmanić et al., 2008). For the present t-PA complexes, the carboxyl, hydroxyl, and amino groups of gelatin and t-PA were considered to contribute to the coordination bond with zinc ions, while the carboxyl groups also contribute to the ionic bond. Taken together, it is highly speculated that t-PA molecules electrostatically interact with gelatin ones to form their complex, and additionally, the complex becomes stable through the coordination bond with zinc ions between the carboxyl, hydroxyl, and amino groups of the two proteins.

The t-PA activity of complexes depended on the molecular weight of gelatin used (Figure 2). This can be explained by the mobility of gelatin molecules. The molecular mobility of lower molecular weight gelatin to interact is high compared with that of higher molecular weight gelatin. Therefore, it is highly conceivable that the t-PA molecules of negative charge interact more with smaller gelatin molecules of positive charge, resulting in the larger molecular size of t-PA complexes (Figure 1). The more interaction would cover t-PA molecules more to suppress the activity to a higher extent (Figure 2A). However, the effect of gelatin molecular weight on the t-PA activity in the presence of BSA was different. The smaller gelatin showed the higher percent t-PA activity (Figure 2B). This suggests that the BSA presence enabled the t-PA–gelatin complexes to dissociate. The electrostatic interaction between smaller gelatin and t-PA molecules is weaker than that of larger gelatin ones. In addition, the smaller gelatin molecules have the higher mobility. As the result, it is likely that small gelatin are readily detached from the t-PA molecules and bound to BSA ones. On the other hand, it is demonstrated that albumin has a high-affinity zinc binding site consisting of His67 and Asn99 from domain I and His247 and Asp249 from domain II (Lu et al., 2008; Stewart et al., 2003). This binding may allow the gelatin and BSA interaction to stabilize, resulting in the increased existence of free t-PA. Based on the stability of t-PA–gelatin complexes in the solution, the

molecular weight of gelatin 100,000 was selected for the t-PA complexes formation.

It is apparent in Figure 4D that the ultrasound irradiation allowed the t-PA activity to recover to the original level. This is because the interaction forces between the t-PA and gelatin molecules in the presence of zinc ions were not so strong to allow dissociation by the ultrasound stimulus. In addition, the ultrasound irradiation did not lose the t-PA activity. In this study, the conditions of ultrasound were a frequency of 1 MHz and an intensity of  $0.75 \text{ W/cm}^2$  in *in vitro* experiments. To date, various ultrasound energy levels ( $0.2\text{--}16 \text{ W/cm}^2$ ) and frequencies (20 kHz–2 MHz) have been used for thrombolysis (Tsivgoulis et al., 2008; Ishibashi et al., 2002). The majority of studies used of 1 MHz both *in vitro* and *in vivo* (Ishibashi et al., 2002), and the frequency is typically lower than that of diagnostic applications. Recently, 1 MHz and  $0.75 \text{ W/cm}^2$  ultrasound significantly enhanced tPA-induced pharmacological lysis of platelet-rich thrombus (recanalization ratio; 90%) with no distal embolization by fragmented thrombi (Kawata et al., 2007). Taken together, the present ultrasound conditions will be safe and effective for sonothrombolysis in the femoral artery model.

It is recognized that some metal ions sometimes show cytotoxicity. Yamamoto et al. evaluated 43 metal salts for cytotoxicity using MC3T3-E1 and L929 cells (Yamamoto et al., 2004).  $\text{K}_2\text{Cr}_2\text{O}_7$ ,  $\text{CdCl}_2$ ,  $\text{VCl}_3$ ,  $\text{AgNO}_3$ ,  $\text{HgCl}_2$ ,  $\text{SbCl}_3$ ,  $\text{BeSO}_4$ , and  $\text{InCl}_3$  are high toxic salts in which  $\text{IC}_{50}$ s are smaller than  $10^{-5} \text{ mol L}^{-1}$  for both or either of the cell lines. The cytotoxicity experiments demonstrated that the complexes with zinc ions showed almost no cytotoxicity even at a gelatin concentration of  $200 \text{ }\mu\text{g/ml}$  (Figure 7). The concentration of t-PA, gelatin, and Zn ions used for complexation in the *in vivo* experiment were  $0.2 \text{ }\mu\text{g/ml}$ ,  $4 \text{ }\mu\text{g/ml}$ , and  $2 \text{ }\mu\text{M}$ , respectively, which is much lower than those of cytotoxicity. At the concentration of t-PA, gelatin, or Zn ions alone, no cytotoxicity was observed. It is conceivable that upon ultrasound exposure, the components of complex and their combinations are released out. I do not think

that the combination of components brings about any cytotoxicity. Therefore, we believe that the t-PA complexes with gelatin and Zn ions after the application of ultrasound irradiation have no cytotoxicity in the *in vivo* experiment.

The t-PA used in this study is mutant t-PA, monteplase (Verstraete, 1999; Inoue et al., 2004), where Cys84 is substituted with Ser in the epidermal growth factor domain of human t-PA. It is reported that the half-life is about 20 min (Verstraete, 1999), which is similar to the present result (Figure 8A). The complexation with the gelatin and zinc ions enabled t-PA to prolong the half-life in the blood circulation. This is because the apparent molecular size of t-PA was reduced by the complexation. Complexing with the gelatin and zinc ions, the apparent molecular size of t-PA became 95 nm (Figure 1). It is reported that the size of long-circulating particles should not exceed 200 nm (Moghimi et al., 2001). In addition, the resistance of t-PA against enzymatic degradation will also be increased by the gelatin complexation. Generally, the biological activity of proteins is protected from enzymatic attack through their modifications with polymer carriers (Sehgal and Srinivasan, 2009). The *in vitro* stability of t-PA in the mouse serum significantly increased by complexing with gelatin and zinc ions with the molecular weight of gelatin 20,000 or higher (data not shown). The complexes administered intravenously retained in the blood circulation for prolonged time periods, while the *in vivo* stability of t-PA was enhanced due to an increased resistance against enzymatic degradation. It has been reported that proteins and peptides encapsulated in poly(lactic/glycolic acid) microparticles are stabilized by their complexing with zinc ions (Schwendeman, 2002; Lam et al., 2001; Carino et al., 2000; Lam et al., 2000; Yamagata et al., 2003). In addition, the coordination bonds with zinc ions contribute to the stabilization of endostatin and  $\alpha$ -crystallin structures (Han et al., 2007; Biswas and Das, 2008).

Drug delivery from alginate microparticles cross-linked with  $\text{Zn}^{2+}$  and  $\text{Ca}^{2+}$  ions has been investigated (George and Abraham, 2006; Jay and Saltzman, 2009; Jay et al., 2008; Colinet et

al., 2009; Chueh et al., 2010). The pH- and UV-sensitive alginate hydrogels cross-linked with calcium ions were reported (Colinet et al., 2009; Chueh et al., 2010). However, ultrasound-responsive carriers modified with metal ions have not been reported. The present study clearly demonstrates that the metal ions modification was effective in the stabilization of ultrasound-responsive drug carrier through the cross-linking. Many ultrasound-triggered drug release with other systems, such as pluronic micelles (Rapoport et al., 2002; Marin et al., 2001), acoustically active liposomes (Daocheng and Mingxi, 2008), the fluoride anion modified with a gelatin nanogel (Frinking et al., 1998), have been reported. Tiukinhoy-Laing *et al.* reported that t-PA loaded-echogenic liposomes increased 1.5 times of clot lysis with the t-PA loaded-liposome alone by ultrasound at 1 MHz and 2 W/cm<sup>2</sup> for 2 min (Tiukinhoy-Laing et al., 2007). In this study, t-PA complexes with gelatin and zinc ions increased about 2 times of t-PA activity compared with the original level by ultrasound at 1 MHz and 0.75 W/cm<sup>2</sup> for 5 min. It is highly speculated that the ultrasound power dissociates the interaction of t-PA and gelatin complexes by cavitation effect which generates shock waves and high-speed fluid microjets (Deckers et al., 2008). From the data comparison, we can say that our delivery system is a sensitive and effective ultrasound-responsive system. For the t-PA complexes at the optimal concentration of zinc ions, the t-PA activity could be recovered by ultrasound irradiation. In the previous study, free t-PA administration with or without ultrasound was effective in recanalization, although there were the adverse effects to be resolved (Kawata et al., 2007). On the contrary, the complexation with gelatin will suppress the adverse effect of t-PA. The t-PA activity is suppressed, but locally recovered upon the ultrasound irradiation around the site necessary.

Combination of a thrombolytic agent and ultrasound enhanced thrombolysis for stroke and acute myocardial infarction (Alexandrov et al., 2004; Eggers et al., 2003; Cohen et al., 2003). In the investigations, it is suggested that the direct transmission of energy and the acoustic

cavitation with low-frequency and high-intensity ultrasound broke clots into small fragments (Rosenschein et al., 1994), promote the penetration of t-PA molecules into the thrombus, the radiation force to facilitate the reformation, and opening of fibrin matrix, and enhance the drug diffusion, and the direct effects on the binding of t-PA to the fibrin clot are the mechanisms of ultrasound on the enhanced thrombolysis (Tiukinhoy-Laing et al., 2007). In the future, we plan further studies of evaluation of the thrombolytic effect of t-PA complexes with or without the local ultrasound irradiation around the infarction site of blood vessels in rabbit and swine thrombosis models.

## CONCLUSION

Novel and promising ultrasound-responsive nano gelatin complexes of t-PA stabilized by zinc ions were designed. This system is safe, simple, and convenient to permit the treatment of acute myocardial infarction while performing diagnosis at the same time even in emergency conditions. Moreover, the delivery system is also applicable to other therapeutic drugs for the medical treatment of various diseases.

## ACKNOWLEDGMENT

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## 1 FIGURE LEGENDS

2 **Figure 1.** The apparent molecular size of gelatin with different molecular weights (final  
3 concentration: 10 mg/ml) and the complexes of t-PA (0.5 mg/ml) and zinc ions (5 mM) : (○)  
4 t-PA complexes with gelatin and zinc ions, (●) gelatin complexes with zinc ions, and (Δ) free  
5 gelatin.

6  
7 **Figure 2.** Change in the t-PA activity by complexing with gelatin (10 mg/ml) and zinc ions (5  
8 mM) as a function of gelatin molecular weight in PBS (A) or BSA solution (B). The percent  
9 t-PA activity was expressed as 100% for free t-PA without zinc ions. \*,  $P < 0.05$ ; significant  
10 against the activity of free t-PA.

11  
12 **Figure 3.** Change in the t-PA activity of complexes with different concentrations of gelatin  
13 ( $M_w = 100,000$ ), t-PA (0.5 mg/ml), and zinc ions (5 mM). The percent t-PA activity was  
14 expressed as 100% for free t-PA without zinc ions. \*,  $P < 0.05$ ; significant against the activity  
15 of free t-PA.

16  
17 **Figure 4.** Change in the t-PA activity of complexes with gelatin ( $M_w = 100,000$ , 10 mg/ml) and  
18 zinc (A), magnesium (B), and calcium ions (C) at different concentrations. (D) Change in the  
19 t-PA activity of t-PA complexes of gelatin and zinc ions by ultrasound irradiation. The percent  
20 t-PA activity was expressed as 100% for free t-PA without zinc ions. \*,  $P < 0.05$ ; significant  
21 against the activity of free t-PA (t-PA).

22  
23 **Figure 5.** The activity of t-PA (0.5 mg/ml) in the presence of zinc ions at different  
24 concentrations. The percent t-PA activity was expressed as 100% for free t-PA without zinc  
25 ions. \*,  $P < 0.05$ ; significant against the activity of free t-PA.

**Figure 6.** Effect of EDTA addition on the t-PA activity of complexes with gelatin ( $M_w = 100,000$ , 10 mg/ml) and zinc ions (5 mM). The percent t-PA activity was expressed as 100% for free t-PA without zinc ions. \*,  $P < 0.05$ ; significant against the activity of free t-PA.

**Figure 7.** (A) Cell viability of t-PA complexes with gelatin ( $M_w = 100,000$ ) and zinc ions. (B) Cell viability of zinc ions. (C) Cell viability of 500  $\mu\text{g/ml}$  of gelatin ( $M_w = 100,000$ ) and 50  $\mu\text{g/ml}$  of t-PA. The percentage of cell viability was expressed as 100% for non-treated cells. \*,  $P < 0.05$ ; significant against the viability of non-treated cells. The dotted line indicates 100% of cell viability.

**Figure 8.** Time profiles of body distribution of t-PA complexes with gelatin ( $M_w = 100,000$ , 10 mg/ml) and zinc ions (5 mM) or free t-PA after intravenous administration. After the intravenous administration of  $^{125}\text{I}$ -labeled t-PA at different forms to mice, the radioactivity remaining was measured to evaluate their body distribution. (A) Retention in the blood circulation: ( $\circ$ ) t-PA complexes with gelatin and zinc ions and ( $\bullet$ ) free t-PA. \*,  $P < 0.05$ ; significant against the radioactivity remaining of free t-PA at the corresponding time. (B) Tissue distribution; t-PA complexes with gelatin and zinc ions (white column) and free t-PA (black column).



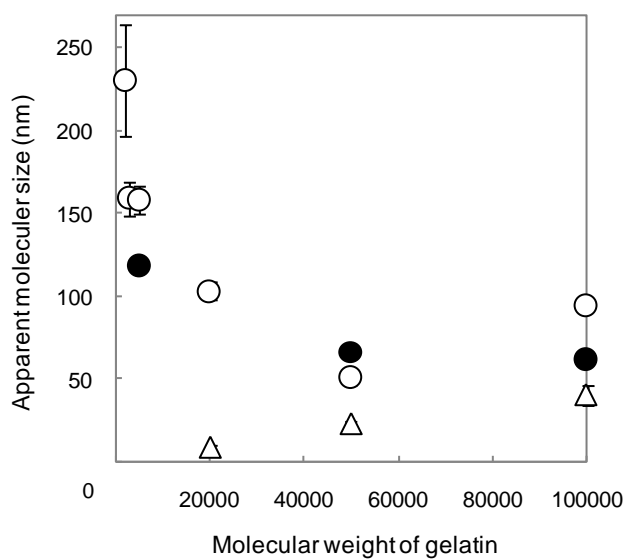


Fig. 1 Uesugi *et al.* ↑

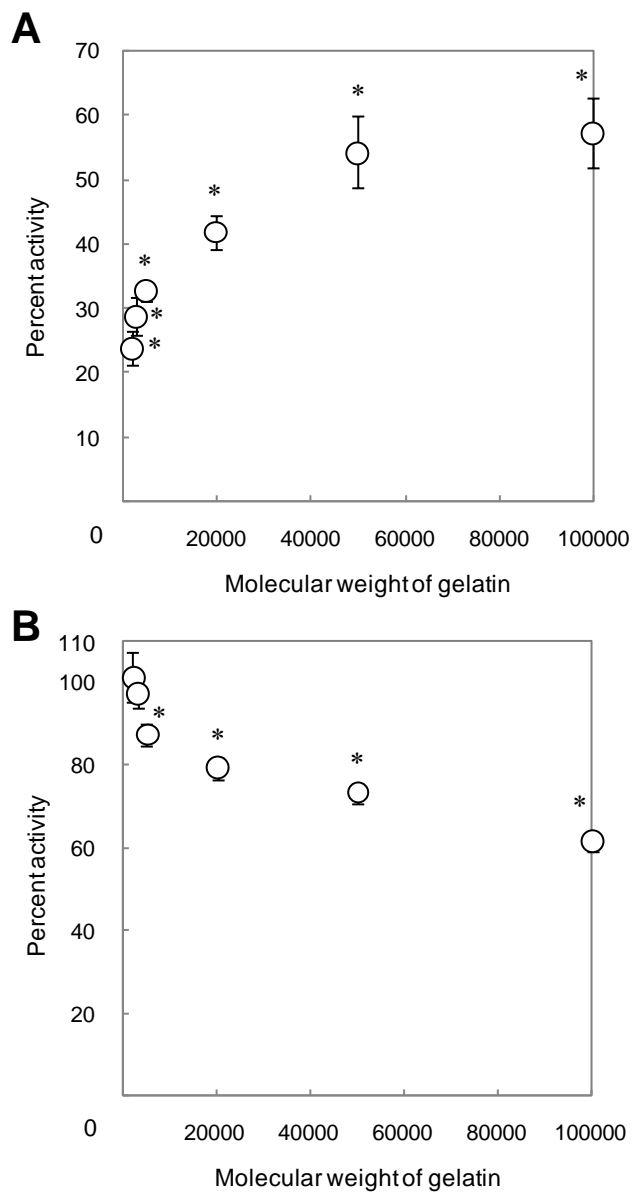


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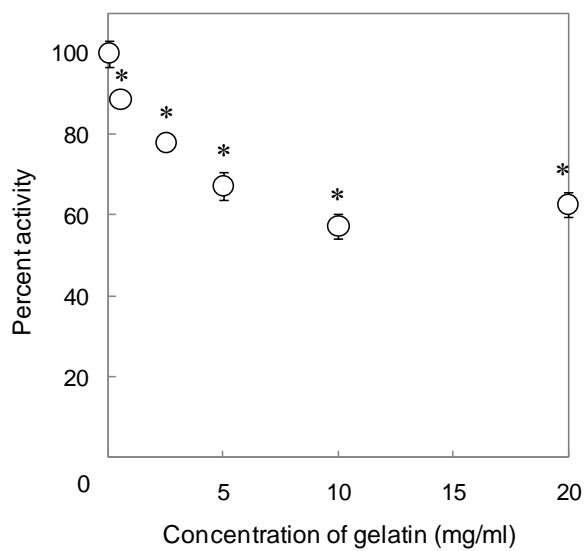


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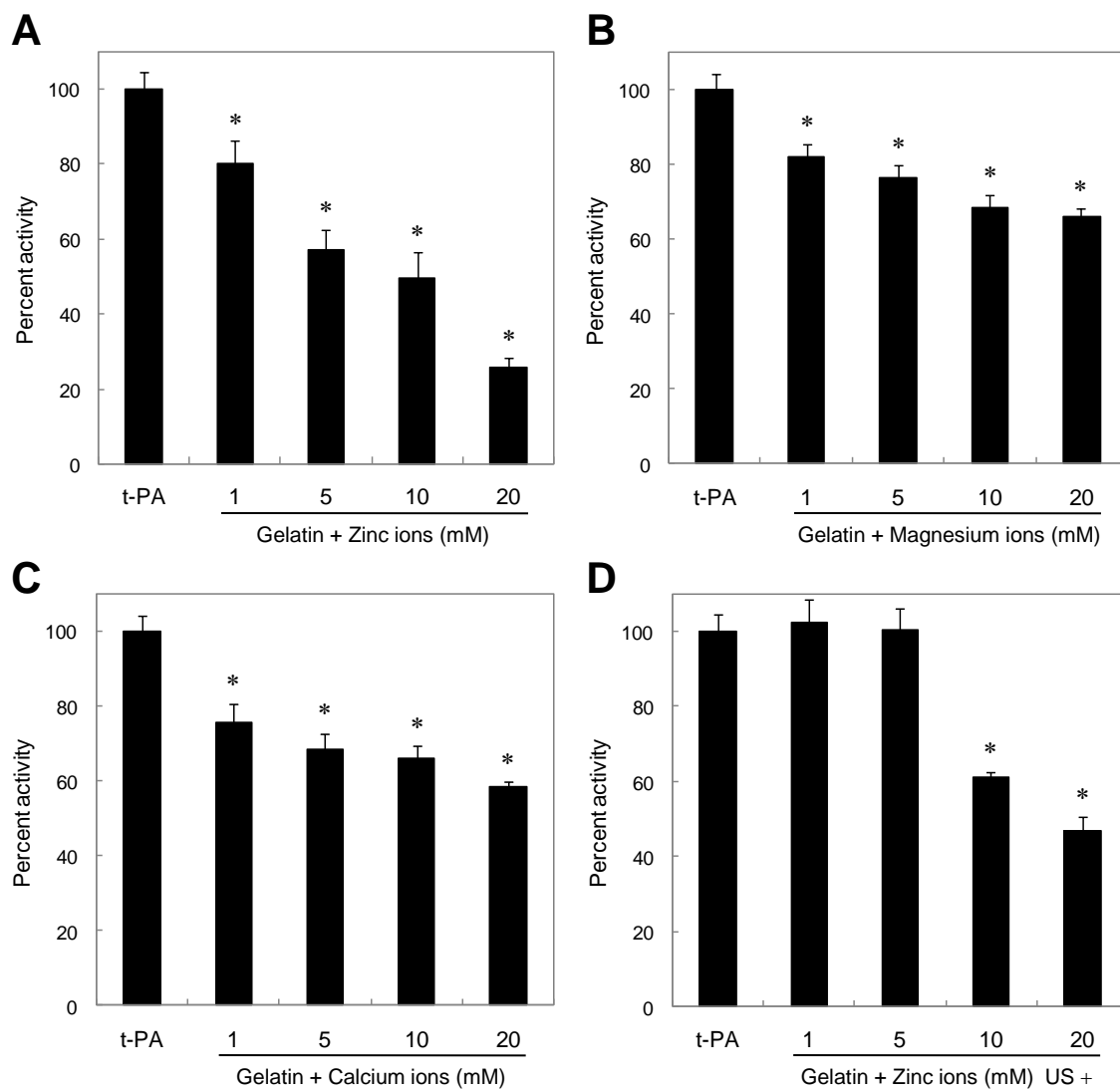


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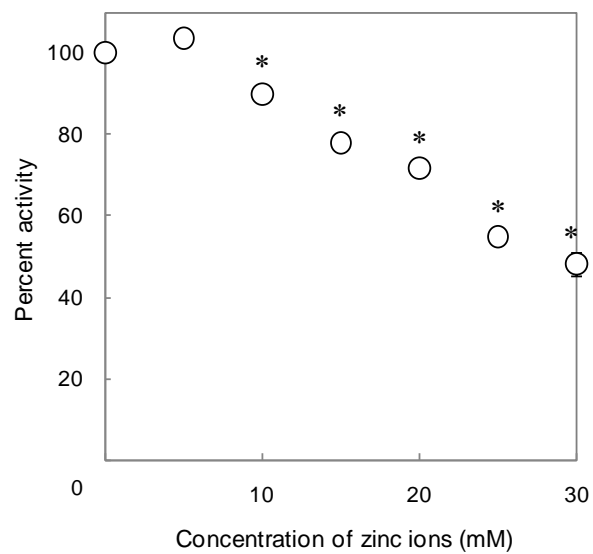


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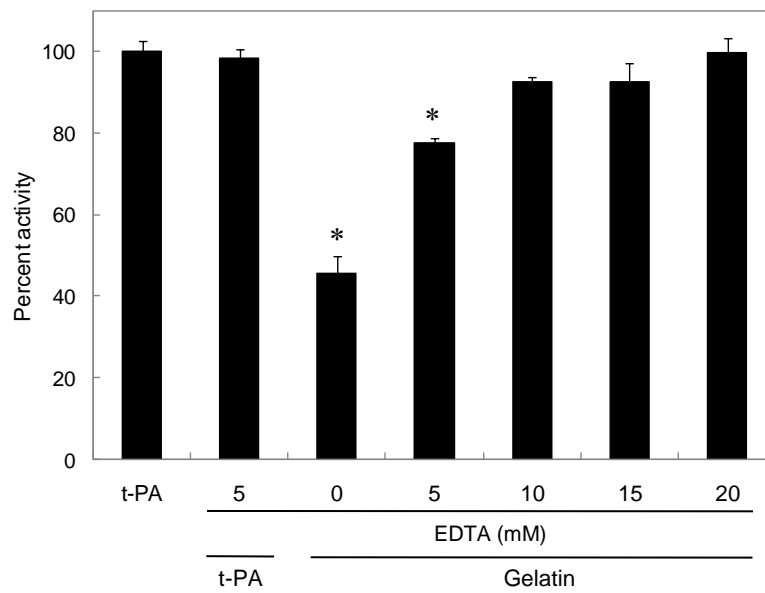


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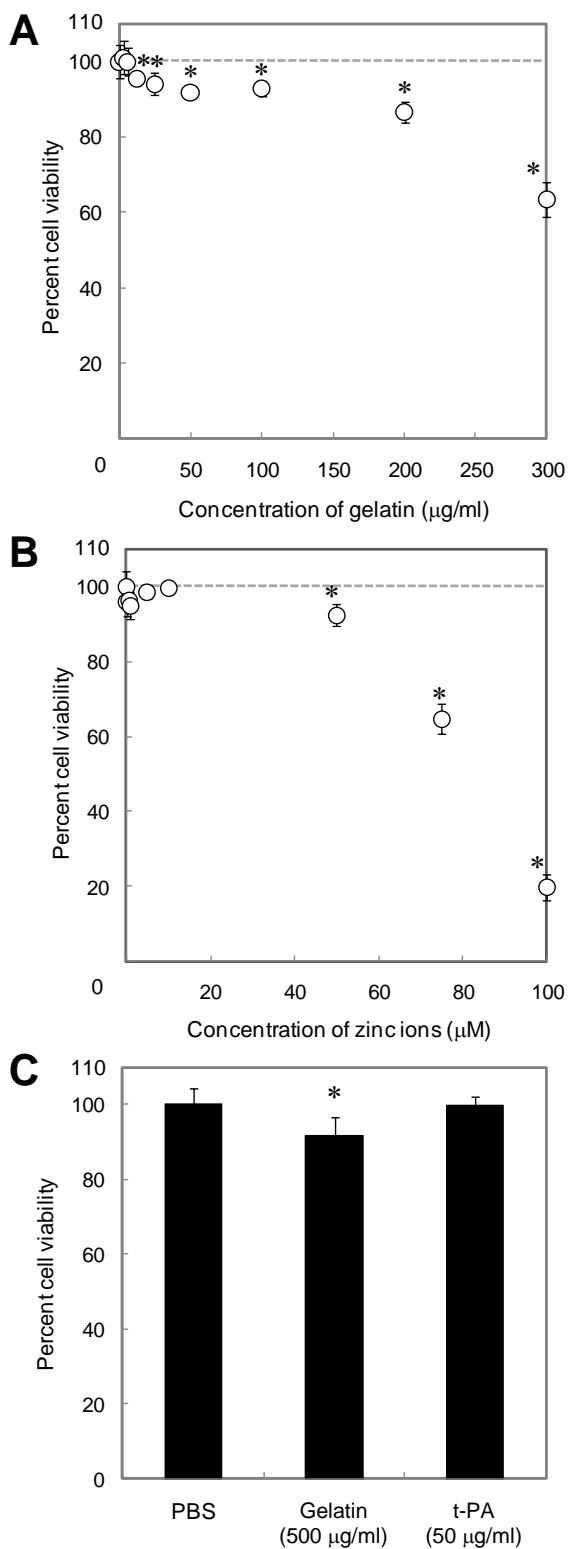


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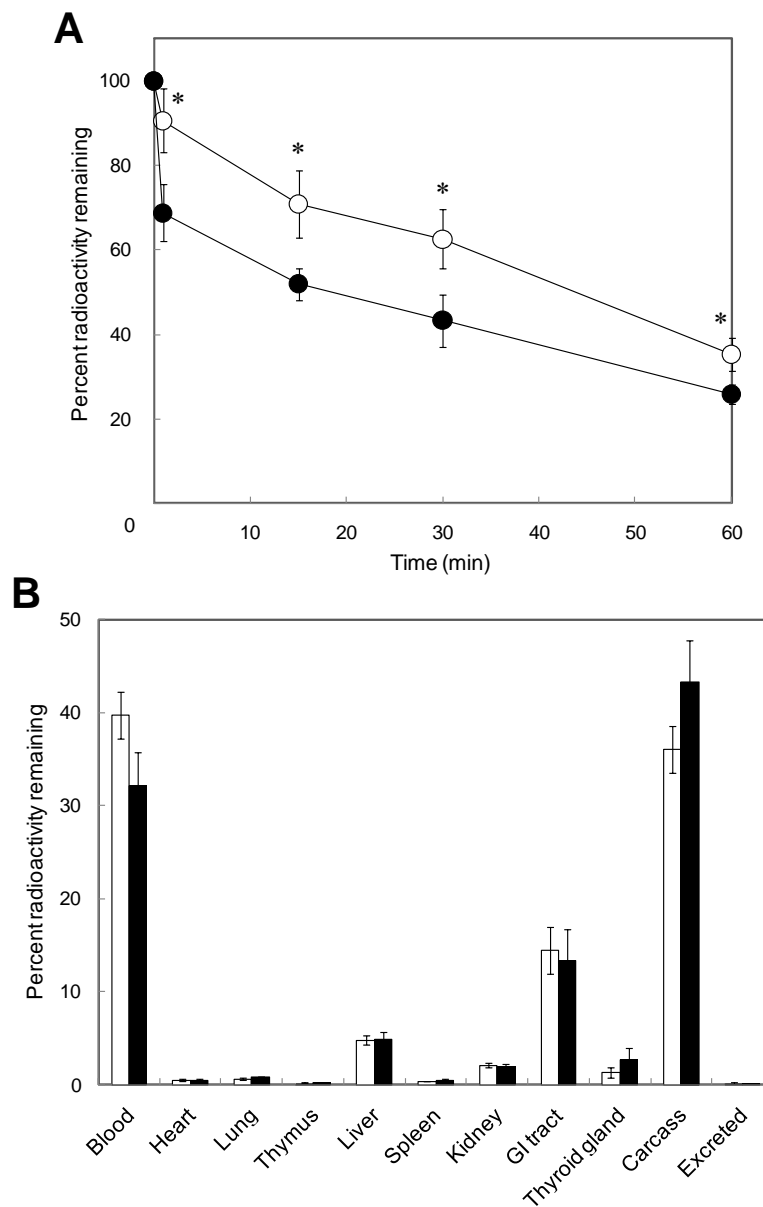


Fig. 8 Uesugi *et al.* ↑